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$$\mathrm{NH_2\,\overline{Cys\text{-}Tyr\text{-}D\text{-}Trp\text{-}Lys\text{-}Thr\text{-}Cys}\text{-}D\text{-}Tyr\text{-}NH_2} \qquad (1)$$

(57) Abstract

The present invention relates to somatostatin analogs which comprises a chemically substituted heptapeptide sequence having the cysteine groups in the 1 and 6 position being linked together to form a disulfide bridge in the monocyclic configuration. Chemical modifications are made at the free amino group of cysteine of the peptide (1). It has been demonstrated that the somatostatin analog peptide can be modified by the addition of isocyanates, isothiocyanates, acid chlorides, chloroformates and glycidyl ethers (epoxides) at the free amino group, at the terminal cysteine resulting in a measurable enhancement of the ability of the chemically modified compounds to bind somatostatin receptors.

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SOMATOSTATIN ANALOGS

BACKGROUND OF THE INVENTION

Somatostatin is a cyclic tetradecapeptide which inhibits release of several pituitary and intestinal factors that regulate cell proliferation, cell motility, and/or secretion including growth hormone, adrenocorticotropin hormone, prolactin, thyroid stimulating hormone, insulin, glucagon, motilin, gastric inhibitory peptide, vasoactive intestinal peptide, secretin, cholecystokinin, bombesin, gastrin releasing peptide, gastrin, thyroid releasing hormone, pancreatic polypeptide, cytokines (e.g., interleukins, interferons), growth factors (e.g., epidermal growth factor, nerve growth factor), and vasoactive amines (e.g., serotonin). Several of these factors are implicated in regulation of normal cell proliferation, as well as in tumor cell proliferation and metastasis.

Native somatostatin has a very short half life in vivo. A large number of novel analogues have been prepared in order to enhance the duration of effect, biological activity and the selectivity of this hormone. A variety of somatostatin peptide analogs have been produced by elimination of amino acids that are not absolutely required for activity and substitution of the native L-amino acids with the corresponding D-amino acid isomers. Thus, some of these analogs are long acting, more potent receptor agonists than native somatostatin, due in part to the resistance of D-amino acids to enzyme degradation. For example, the synthetic somatostatin analog octreotide acetate, which has the amino acid sequence D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol) is more potent

than native somatostatin in inhibition of growth factor release. Bauer et al. U.S. Patent No. 4,395,403.

SUMMARY OF THE INVENTION

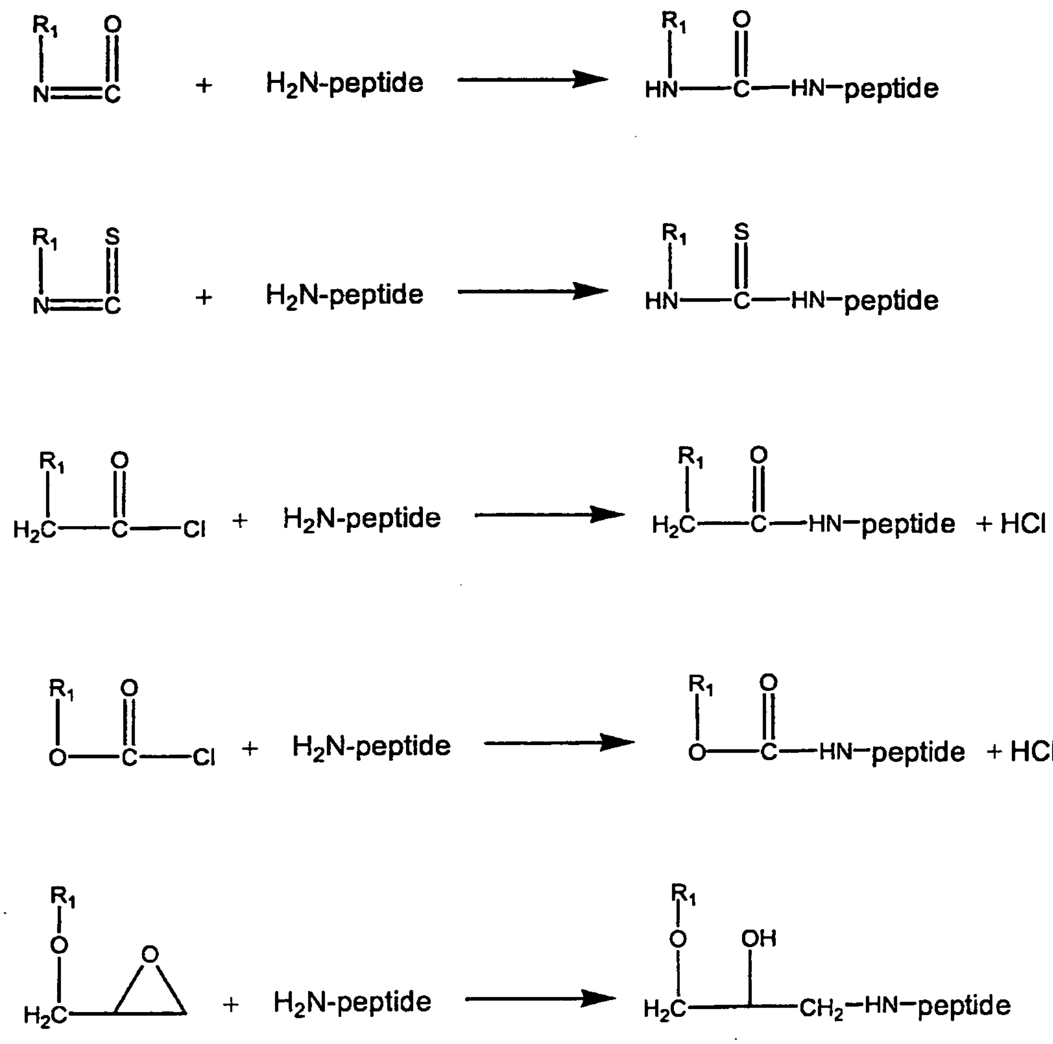
The present invention provides novel chemically modified somatostatin analogs, structural derivatives of native somatostatin which bind a somatostatin receptor. Analogs include both antagonists and agonists of somatostatin activity.

More particularly, the present invention provides a somatostatin analog which comprises a chemically substituted heptapeptide sequence having the cysteine groups in the 1 and 6 position being linked together to form an disulfide bridge in the monocyclic configuration. Chemical modifications are made at the free amino group of cysteine of the peptide below

$$\text{A-NH - }\overbrace{\text{Cys-Tyr-D-Trp-Lys-Thr-Cys}}\text{-D-Tyr-NH}_2 \qquad \text{I}$$

It has been demonstrated that the somatostatin analog peptide can be modified by the addition of isocyanates, isothiocyanates, acid chlorides, chloroformates and glycidyl ethers (epoxides) at the free amino group, at the terminal cysteine resulting in a measurable enhancement of the ability of the chemically modified compounds to bind somatostatin receptors.

The following synthetic reaction schemes are used to generate the chemically modified peptides.



BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graph which illustrates that peptide 3502 suppresses secretion of growth hormone.

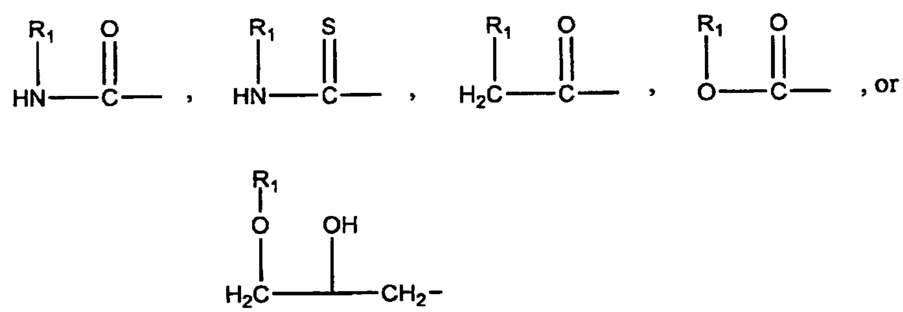
Figure 2 is a graph which shows that orally administered peptide 3502 prevents normal pulsatile secretion of growth hormone.

DETAILED DESCRIPTION OF THE INVENTION

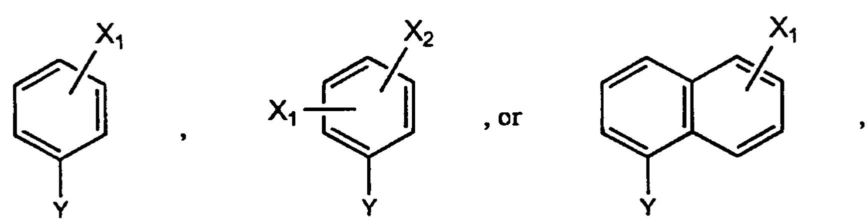
The compounds of this invention are cyclic heptapeptide analogs of somatostatin having the general structure of Formula I

A-NH - Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2 I

wherein A is

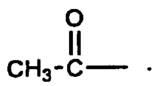


and R1 is C1-C4 alkyl, adamantyl,



Y is a bond, C1-C4 alkenyl, C=O, or SO_2 ; and

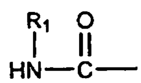
X_1 and X_2 are independently, flourine, chlorine, bromine, iodine, C1-C4 alkyl, NO_2 or



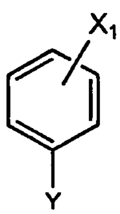
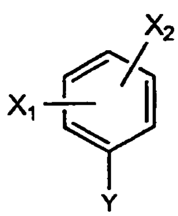
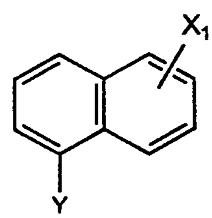
The chemical modifications of Formula I are generated at the free amino group of the terminal cysteine moiety of the heptapeptide sequence.

Preferred compounds of this invention include compounds of Formula I:

wherein A is

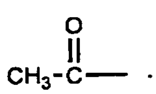


R1 is C1-C4 alkyl, adamantyl,

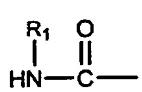
 ,  , or  ,

Y is a bond, C1-C4 alkenyl, C=O, or SO_2 ; and

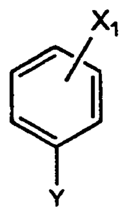
X_1 and X_2 are independently, flourine, chlorine, bromine, iodine, C1-C4 alkyl, NO_2 or



Another preferred compound of this invention of Formula I was the following structure wherein A is



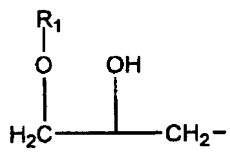
R_1 is



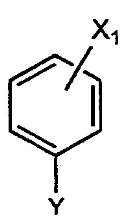
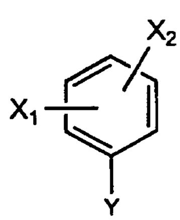
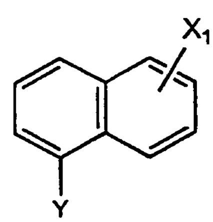
Y is CH_2 and

X_1 is hydrogen.

Other preferred somatostatin analogs are compounds of Formula I

wherein A is 

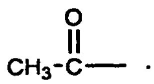
R1 is C1-C4 alkyl, adamantyl,

 ,  , or  ,

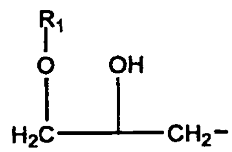
Y is a bond, C1-C4 alkenyl, C=O, or SO_2 ; and

X_1 and X_2 are independently, flourine, chlorine, bromine, iodine, C1-C4 alkyl,

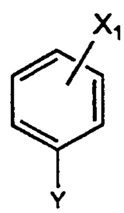
NO_2 or



Another preferred somatostatin analog of this invention is a compound of Formula I

wherein A is  and

R_1 is



X_1 is hydrogen, and

Y is a bond.

The invention features compounds, compositions and methods for the treatment of diseases in mammals associated with increased production or secretion of any factor or factors which can be regulated by somatostatin, including but not limited to growth hormone, insulin, glucagon and pancreatic exocrine secretion.

The compounds can be administered in the dosages used for somatostatin or, because of their greater potency, in smaller dosages. The compounds of the invention can be used for the treatment of cancer, particularly growth hormone- or growth factor-dependent cancer (e.g., bone, cartilage, pancreas, prostate, or breast), acromegaly and related hypersecretroy endocrine states, or of bleeding ulcers and in those suffering from

pancreatitis or diarrhea. The compounds can also be used in the management of diabetes and to protect the liver of patients suffering from cirrhosis or hepatitis. The compounds can also be used to treat Alzheimer's disease and as gastric cytoprotective compounds for ulcer therapy. The compounds will also be useful in treating diabetes-related retinopathy, nephropathy and vascular disease. The anti-cancer activity of the compounds may be related to their ability to antagonize the actions of cancer-related growth factors such as epidermal growth factor, insulin-like growth factor (IGF-1), or vasoactive endothelial growth factor (VEGF).

The analogs can be made available in the form of pharmaceutically acceptable salts or complexes. Examples of therapeutically acceptable acids for the formulation of salts of the somatostatin analogs are inorganic acids, such as hydrochloric acid, sulfuric acid, phosphoric acid, and the organic lactic, maleic, citric, succinic, benzoic, salicylic, toluensulfonic acids. Complexes are compounds of Formula I formed by the addition of organic salts or hydroxides such as Ca and Zn salts or the addition polymeric organic materials, such as tannic acid or carboxymethyl cellulose.

In other preferred embodiments, a therapeutically effective amount of the somatostatin analog or pharmaceutically acceptable salt or complex thereof are combined with a pharmaceutically acceptable carrier substance (e.g., magnesium carbonate, lactose, a phospholipid or mannitol) to form a pharmaceutical composition. Examples of methods of administration of the therapeutic reagent of the pharmaceutical composition thereof include a pill, tablet, capsule or liquid for oral administration. The pharmaceutical composition can also be administered as an ointment, gel, cream or lotion

for application to the skin, or as a solution capable of being administered intravenously, parenterally, subcutaneously, transmucosally, intranasally or intraperitoneally in an appropriate buffer if necessary. The solid forms of this therapeutic composition can be coated with a substance capable of protecting the modified peptide from digestion by gastric acid in the stomach for a period of time sufficient to allow the composition to pass undisintegrated into the small intestine. The therapeutic composition can be administered via a sustained release formulation or a dermal patch. The descriptions are provided as examples and are not meant to limit the possibilities of therapeutic compositions or methods of administration of the somatostatin analogs.

Examples

The cyclic heptapeptide NH_2 Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2 was purchased from Polypeptide Laboratories. The reagents used to modify the heptapeptide are widely available from commercial sources.

In accordance with the present invention, numerous modified peptides have been synthesized according to the synthetic schemes outlined below.

1) Reactions of isocyanate with the heptapeptide of Formula I:

The heptapeptide (as a trifluoroacetic acid salt) containing Boc-Lys was suspended in anhydrous acetonitrile to yield a 1 mM concentration. Fifty to 100 µl of

this mixture was placed in a microcentrifuge tube. One equivalent of triethylamine (100 mM in acetonitrile) was added with mixing, followed by the addition of 1 equivalent of the isocyanate (100 mM in acetonitrile). The reactions with the isocyanates were incubated at room temperature for 60 to 120 min. Ten μl of water was added. The solvent was removed by evaporation under vacuum. The chemically modified peptides were than purified by reverse phase HPLC.

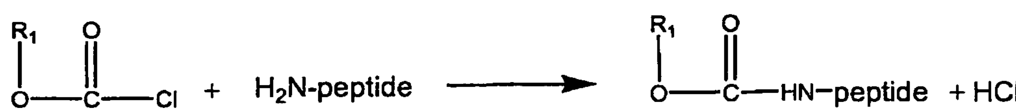
2) Reactions with isothiocyanates:

The heptapeptide (as a trifluoroacetic acid salt) containing Boc-Lys was suspended in anhydrous acetonitrite to yield a 1 mM concentration. Fifty to 100 μl of this mixture was placed in a microcentrifuge tube. One equivalent of triethylamine (100 mM in a acetonitrile) was added with mixing, followed by the addition of one equivalent of isocyanate (100 mM in acetonitrile). The reactions with the isothiocyanates were incubated at room temperature for 18 hrs. The solvent was removed by evaporation under vacuum. The chemically modified peptides were then purified by reverse phase HPLC.

3) Reactions with acid chlorides:

The heptapeptide (as a trifluoroacetic acid salt) containing Boc-Lys was suspended in anhydrous acetonitrile to yield a 1 mM concentration. Fifty to 100 μl of this mixture was placed in a microcentrifuge tube. Two equivalents of triethylamine (100 mM in acetonitrile) was added with mixing, followed by the addition of 1 equivalent of an acid chloride (100 mM in acetonitrile). The reactions with the acid chlorides were incubated at room temperature for 60-120 min. The solvent was removed by evaporation under vacuum. The chemically modified peptides were then purified by reverse phase HPLC.

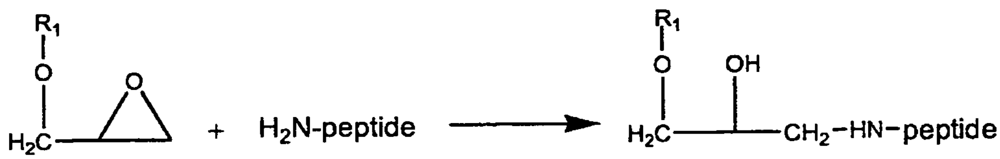
4) Reactions with chloroformates:



The heptapeptide (as a trifluoroacetic acid salt) containing Boc-Lys was suspended in anhydrous acetonitrile to yield a 1 mM concentration. Fifty to 100 μl of this mixture was placed in a microcentrifuge tube. Two equivalents of triethylamine (100 mM in acetonitrile) was added with mixing, followed by the addition of 1 equivalent of a chloroformate (100 mM in acetonitrile). The reactions with the

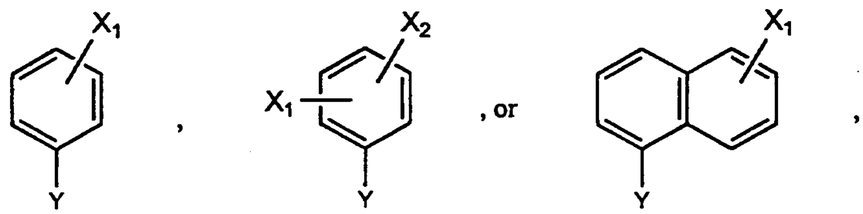
chloroformates were incubated at room temperature for 60-120 min. The solvent was removed by evaporation under vacuum. The chemically modified peptides were then purified by reverse phase HPLC.

5) Reactions with glycidyl ethers (epoxides):



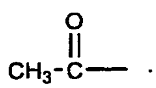
The heptapeptide (as a trifluoroacetic acid salt) containing Boc-Lys was suspended in anhydrous methanol to yield a 1 mM concentration. Fifty to 100 µl of this mixture was placed in a microcentrifuge tube. One equivalent of triethylamine (100 mM in acetonitrile), followed by the addition of 1 equivalent of a glycidyl ether (100 mM in methanol). The reaction was incubated at 65° C for 6-8 hrs. The solvent was removed by evaporation under vacuum. The chemically modified desired peptides were purified by reverse phase HPLC.

In the resulting modified peptides, R_1 is C1-C4 alkyl, adamantyl,



Y is a bond, C1-C4 alkenyl, C=O, or SO_2 ; and

X_1 and X_2 are independently, fluorine, chlorine, bromine, iodine, C1-C4 alkyl, NO_2 or



<u>HPLC purification of chemically modified amino acids.</u> The dried reaction product was resuspended in 15µl of 100% of trifluoroacetic acid or 30 µl of 50% trifluoroacetic acid over a period of 5-10 minutes. The volume was brought to 100µl with 50% acetonitrile and the mixture was injected onto a C18 reverse phase HPLC column. The desired product was eluted from the column in a linear gradient of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile (B) progressing from 5% B at initial conditions to 60% B over 40 minutes. The elution position of the desired product was monitored by UV absorbance and the desired peak was collected by

hand in polypropylene tubes as it eluted from the UV detector. The eluent was dried under vacuum then used for binding and bioassays.

A variety of tissues and cancers express somatostatin receptors. Five human somatostatin receptors (hsst1, hsst2, hsst3, hsst4, hsst5) have been identified and cloned. (Patel, Y.C., Life Sciences, Vol. 57, No. 13, pp. 1249-1265, 1995). Expression of these five receptor subtypes varies with tissue types. Somatostatin receptor subtype 2 is expressed on a wide variety of tumor types.

Receptor Binding Assays

Cell culture. CHO-K1 cells were grown as monolayers in Dulbecco's Modified Eagle's medium (DMEM, Mediatech, Washington DC) supplemented with 10% fetal calf serum, non-essential amino acids, 2mM glutamine, 1mM pyruvate and 500 mg/mL gentamycin in 5% CO2 at 37°C.

Expression of hsst in CHO-K1 cells. For binding studies, CHO-K1 cell lines stably expressing hsst were created and propagated. The predicted coding region of each hsst was generated by PCR from human genomic DNA and oligonucleotides corresponding to the coding region 5' and 3' ends as primers. The DNA fragment generated by PCR contained a Hind III restriction site at the 5' end and a Not 1 restriction site at the 3' end. The fragment was digested with these two restriction enzymes and directionally subcloned into the Hind III/Not 1 sites of the mammalian expression vector pCDNA1. The identity of each insert was verified by DNA sequencing. The construct was co-transfected with pSV2neo into a CHO-K1 cell line using the calcium phosphate protocol. Stable transfectants were selected using 400 mg/mL G418 and maintained in supplemented DMEM. After an initial ligand binding screen, one stable clone for each sst was chosen for all subsequent experiments.

Preparation of Plasma Membranes. CHO/sst cells grown on 100mm tissue culture dishes were washed with ice cold PBS then scraped into 5ml of 50mM HEPES, pH 7.4-5mM $MgCl_2$ - 200 KIU/mL aprotinin - 2mg/mL PMSF and 2 mg/mL bacitracin (homogenization buffer). After a 15 min. incubation at 4°C, the cells were homogenized on ice using a Brinkman Polytron (setting 5, 15 sec) then re-homogenized with a hand held homogenizer (6 strokes). After centrifugation at 500 x g for 5 min. at 4°C, the supernatant was centrifuged again at 12,000 x g for 25 minutes at 4°C. The final pellet was resuspended in homogenization buffer. Protein content was measured using the bicinchoninic acid protein assay using BSA as a standard.

Preparation of 96-well plates. Costar 96-well strip plates (cat. no. 9102) were coated with poly-l-lysine by incubating each well in 50µL 100mg/mL poly-l-lysine for 1hr at 22°C. Excess liquid was removed and the wells were air dried. Membranes (10mg/well) were added in 20mM Hepes pH 7.6 followed by incubation overnight at 4°C to evaporate all liquid. Non-specific binding sites were blocked by incubating each well in 50µL homogenization buffer supplemented with 1% BSA (incubation buffer) for 30 min at RT. Binding assays were performed after removal of excess liquid.

[^{125}I-Tyr^{11}] SS14 binding. For receptor binding studies, membranes were incubated at 22°C with 0.03nM [^{125}I-Tyr^{11}] SS14 (obtained from Amersham) with or without test compounds each at concentrations ranging from 10^{-10} M to 10^{-6} M in 50µL incubation buffer. After a 1 hour incubation at 22°C, excess liquid was removed by gently tapping plates onto absorbent filter paper. Membranes were washed twice with 100µL ice cold incubation buffer and radioactivity in each well was determined. Specific binding was defined as the difference between the amount of [^{125}I-Tyr^{11}] SS14 bound in the absence and presence of 1µM unlabeled SS14. Ki was determined using software programs Ligand or Prism.

The purified peptides were tested for binding to one or more of the five human somatostatin receptor subtypes. Table 1 lists the results of receptor binding studies for a number of peptides assayed against the five human somatostatin receptor subtypes. The data of Table 1 indicates the chemical modifications change the binding affinity of the parent heptapeptide to the various receptor subtypes.

TABLE 1

			Ki's Human Receptors (nM)				
#	Core Peptide Seq.	Reacting Compound	sst1	sst2	sst3	sst4	sst5
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	none		48.00			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	1-Adamentyl Isocyanate		2.65			
3502	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Benzyl Isocyanate		0.65	31.4	1566	3.5
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Chlorophenyl Isocyanate	2000	0.90	31.5	1411	6.32
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Methoxyphenyl Isocyanate	188	0.59	8.87	203	2.26
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	(R)-(+)-alpha-Methylbenzyl isocyanate	1940	0.41	21.5	1104	7.22
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	(S)-(-)-alpha-Methylbenzyl isocyanate	522	0.81	21	361	4.25
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	(R)-(-)-1-(1-Naphthyl)ethyl isocyanate					
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	(S)-(+)-1-(1-Naphthyl)ethyl isocyanate		147.00			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Nitrophenyl isocyanate	604	0.84	23.4	959	4.19
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Phenyl isocyanate	778	0.44	12.1	769	3.88
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	glycidyl 2-methylphenyl ether					
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	none	1000	0.33	2	1000	8

	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	1,2 epoxy-3-phenoxypropene		148			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	[2,3 Epoxypropyl] benzene		36			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Chlorophenyl glycidyl ether		7.7			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-tert-Butyl phenyl glycidyl ether		12			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	glycidyl 4-methoxyphenyl ether					
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Benzenesulfonyl isocyanate		1.9			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Benzoyl isocyanate		3.4			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Benzoyl isothiocyanate		2.9			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Benzyl isothiocyanate		216			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Chlorobenzenesulfonyl isocyanate		3.7			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	p-Toluenesulfonyl isocyanate		2.1			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Fluorophenyl isocyanate	1093	1.34	38.6	1664	10.1
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Phenethyl Isothiocyanate		103			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	p-Tolyl isocyanate	823	0.62	20.4	1120	6.36
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Trifluoro-p-tolyl isocyanate		3.3			
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Bromophenyl isocyanate	1206	1.05	31.5	1204	8.64
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	2-Phenylphenyl isocyanate	454	0.92	50	799	10.4
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Phenethyl isocyanate					
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	2,6-Dimethylphenyl isocyanate					
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Phenoxyphenyl isocyanate		1.9			
3533	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Acetylphenyl isocyanate	1522	0.21	29.9	1147	5.65
	Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-n-Butylphenyl isocyanate		2.6			

Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Chloro-2-methylphenyl isocyanate		1			
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	2,4-Dichlorobenzyl isocyanate		1.2	14.4	591	1
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	2,3-Dimethylphenyl isocyanate		0.69	8.4	353	3.9
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	2,4-Dimethylphenyl isocyanate		0.7	6.9	251	2.8
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	2,5-Dimethylphenyl isocyanate		0.62	8.1	284	2
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	3,4-Dimethylphenyl isocyanate		0.77	11.7	105	3.9
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	3,5-Dimethylphenyl isocyanate	1334	0.43	10.7	561	3.03
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	4-Ethylphenyl isocyanate		0.81	12.1	274	3.1
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	p-Toluoyl chloride		1.35	17.6	340	1.7
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Cinnamoyl chloride		1.01	19.3	432	4.5
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Phenyl chloroformate		2.58			
Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2	Benzyl chloroformate					
Cys-Tyr-D-Trp-Lys-Val-Cys-D-Tyr-NH_2	none		18			
Cys-Tyr-D-Trp-Lys-Val-Cys-D-Tyr-NH_2	benzyl isocyanate		1.8			
Cys-Tyr-D-Trp-Lys-Val-Cys-D-Tyr-NH_2	Phenyl isocyanate		4.1			
Cys-Tyr-D-Trp-Lys-Val-Cys-D-Tyr-NH_2	4-Acetylphenyl isocyanate		1.9			

Bioactivity Assays

The in vivo biological activity of modified peptides was determined by evaluating their inhibitory potency on pituitary growth hormone (GH) release in sodium pentobarbital-anesthetized rats. The pentobarbital treated rat is a well characterized and frequently used model for studying GH secretory dynamics (see K. Chihara, A. Armura and AV Schally 1979 Endocrinology 104 1434).

Dose-Response Studies: Adult male Sprague-Dawley rats weighing 250-300g with jugular vein cannulas were obtained from Zivic-Miller Labs, Zelienople, PA. On the day of assay, the rats were anesthetized with sodium pentobarbital (60mg/kg of body weight, administered i.p.). Thirty minutes later, the animals were injected iv. with saline or test compound at doses ranging from 0.1 to 30 μg/kg. Blood samples (250 μL) were drawn from the jugular vein cannula 10 min prior to test compound injection (baseline) and 5, 15, 30, 45 and 60 minutes after injection. The plasma was separated and assayed for GH by RIA using material supplied by the National Hormone and Pituitary Program, and for glucagon and glucose using commercially available reagents. To prevent hemodynamic disturbances, the red blood cells were resuspended in normal saline and returned to the animal. Figure 1 illustrates the dose response of peptide 3502 at three dosage levels 5μg/kg, 2.5 μg/kg, and 1 μg/kg. At each dosage, the peptide was shown to be effective in suppressing production of growth hormone.

Time-Course Assay: Groups of cannulated rats were treated with sodium pentobarbital as in the dose-response assay. Thirty minutes later animals were injected via the jugular cannula with saline or test compound at the minimum dose giving maximal GH inhibition. Sodium pentobarbital at half the initial dose was given at 60- to 90-minute intervals to maintain anesthesia. Blood (250 μL) was collected from the jugular vein at approximately 15, 30, 60, 120, 180, and 240 min. after the injection of test compound and treated as described above. Data from the time-course assay are shown in Table 2. The results of this experiment demonstrates that compounds 3502 and 3533 block the ability of arginine to stimulate glucagon secretion, mimicking a normal function of somatostatin. The alpha cells which secrete glucagon are known to express the SST_2 receptor; thus this activity of the peptides is consistent with their selectivity for the SST_2 receptor.

The data also show that the peptides do not cause hypoglycemia, either alone or in combination with arginine.

Time	Treatment	Assay: Glucagon (pg/ml) mean	sem	GH (ng/ml) mean	sem	Glucose (mg/dl) mean	sem
-35 min	pentobarbital 70mg/kg						
-15 min		47.1	8.6	255.4	175.4	153.1	8.1
-10 min	saline						
-5 min		40.6	4.5	64.5	32.3	140.4	8.8
0 min	L-arg. ,400mg/kg						
5min		102.1	7.2	43.9	18.4	160.3	10.9
10 min		82.8	7.7	28.4	10.9	162.0	14.4
15 min		63.7	0.9	26.5	10.8	136.7	13.8
30min		49.7	6.1	46.0	14.4	103.1	11.9
45 min		45.1	3.6	57.4	13.8	91.9	5.1
60 min		41.2	4.4	59.1	4.2	103.9	3.0
75 min		42.7	10.8	54.3	8.2	107.2	2.6

Time	Treatment:						
-35 min	pentobarbital 70mg/kg						
-15 min		50.2	2.0	283.7	208.8	152.5	1.9
-10 min	MS3502, 5ug/kg						
-5 min		43.1	8.3	72.1	31.2	143.7	3.2
0 min	L-arg. ,400mg/kg						
5min		76.2	8.8	24.3	8.1	162.4	4.0
10 min		62.8	12.6	12.7	3.5	150.3	8.3
15 min		38.9	18.0	9.7	2.4	128.4	7.7

30min		29.9	11.8	6.4	0.8	89.9	6.5
45 min		34.1	10.5	13.4	5.9	75.9	8.0
60 min		34.6	10.0	22.5	11.3	88.0	7.0
75 min		37.5	4.6	27.0	10.7	98.7	3.2

Time	Treatment:						
-35 min	pentobarbital 70mg/kg						
-15 min		50.1	8.4	284.3	230.8	157.7	7.1
-10 min	MS3533, 5ug/kg						
-5 min		40.4	7.5	79.0	46.9	150.0	2.3
0 min	L-arg. ,400mg/kg						
5min		73.2	11.6	34.8	23.5	162.2	8.7
10 min		65.2	10.2	28.1	18.0	156.5	1.2
15 min		46.1	4.0	20.3	10.5	138.6	6.0
30min		38.0	2.4	12.2	3.6	100.9	4.5
45 min		37.5	6.1	12.5	3.8	88.9	1.7
60 min		39.2	8.2	14.5	7.5	88.8	8.4
75 min		46.3	7.9	15.1	6.8	103.4	14.6

Time	Treatment:						
-35 min	pentobarbital 70mg/kg						
-15 min		40.8	5.7	89.9	45.7	153.4	7.7
-10 min	MS3502, 1ug/kg						
-5 min		35.1	3.2	41.9	16.4	145.7	0.9
0 min	L-arg. ,400mg/kg						

5min		95.6	11.6	26.1	9.9	161.6	5.9
10 min		72.2	4.2	21.2	5.7	162.4	14.0
15 min		50.9	3.7	18.8	5.7	126.8	8.8
30min		43.9	6.3	39.5	18.4	91.9	5.4
45 min		43.5	6.0	43.4	5.9	77.7	3.0
60 min		36.2	4.2	65.5	14.9	90.1	4.8
75 min		36.6	7.8	60.4	17.7	102.3	0.1

Time	Treatment:						
-35 min	pentobarbital 70mg/kg						
-15 min		49.5	4.9	137.0	73.1	157.2	1.5
-10 min	MS3533, 1ug/kg						
-5 min		41.7	6.1	61.8	21.0	144.6	6.8
0 min	L-arg. ,400mg/kg						
5min		111.0	17.5	32.2	6.4	159.2	1.4
10 min		77.3	10.2	23.5	5.1	151.5	3.1
15 min		59.8	3.2	19.3	1.0	128.3	4.1
30min		48.8	4.4	40.6	13.7	93.5	6.9
45 min		47.9	5.7	53.1	21.8	89.6	1.6
60 min		39.6	8.4	45.3	14.3	99.2	9.0
75 min		33.8	17.0	24.7	12.4	76.0	38.2

Time	Treatment:						
-35 min	pentobarbital 70mg/kg						
-15 min		57.0	3.76				
-10 min	MS3502, 5ug/kg			46.9	10.2	170.1	12.3

-5 min		53.3	2.40				
0 min	saline						
5min		50.0	4.43	22.9	3.2	144.0	8.1
15 min		49.5	3.52	12.0	1.6	128.5	5.1
30min		50.5	2.72	8.7	1.3	128.7	17.8
45 min		64.8	10.23	19.2	11.9	159.3	32.9
60 min		64.3	3.82	71.0	64.4	184.0	50.2
75 min		62.5	5.48				
90 min		80.3	16.21				
105 min		87.5	10.77				
120 min		69.3	10.74				

(nb: this last series of animals has different animals for the glucagon vs. GH & glucose)

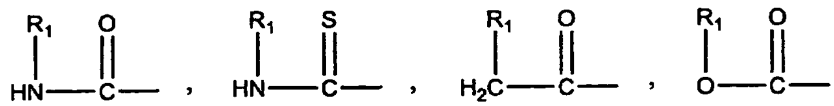
Oral activity: Adult male Sprague-Dawley rats weighing 250-300g with jugular vein and gastric cannulas were obtained from Zivic-Miller Labs, Zelienople, PA. On the evening prior to assay, rats were given 5 gram of food to eat with free access to water. On the day of assay, the rats were anesthetized with sodium pentobarbital (60mg/kg of body weight, administered i.p.). Thirty minutes later, the animals were injected through the gastric cannula with saline or test compound at doses ranging from 0.1 to 30 μg/kg in a total volume of 200 μL. Sodium pentobarbital at half the initial dose was given at 60- to 90-minute intervals to maintain anesthesia. Blood (250 μL) was collected from the jugular vein at approximately 15, 30, 60, 120, 180, and 240 min. after the injection of test compound and treated as described above. In Figure 2 the graph representing oral saline illustrates the cyclic increase and decrease of growth hormone levels during normal secretion. The graph representing peptide 3502 shows that the peptide, orally administered, prevents the normal secretion of growth hormone.

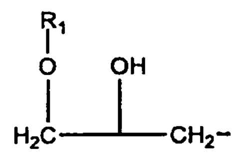
WHAT IS CLAIMED IS:

1. A modified heptapeptide of Formula I

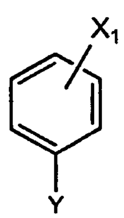
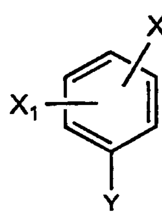
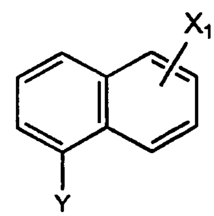
A-NH - Cys-Tyr-D-Trp-Lys-Thr-Cys-D-Tyr-NH_2 I

wherein A is

 , or

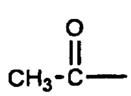


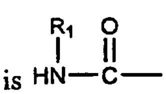
and R_1 is C1-C4 alkyl, adamantyl,

 ,  , or  ,

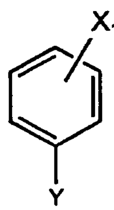
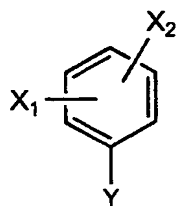
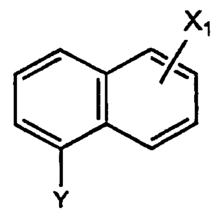
Y is a bond, C1-C4 alkenyl, C=O, or SO_2 ; and

X_1 and X_2 are independently hydrogen, fluorine, chlorine, bromine, iodine, C1-C4 alkyl, NO_2 or

 .

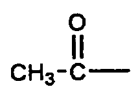
2. The modified heptapeptide of Claim 1 wherein A is 

and R_1 is C1-C4 alkyl, adamantyl,

 ,  , or  ,

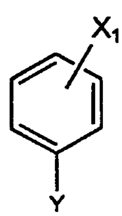
Y is a bond, C1-C4 alkenyl, C=O, or SO_2; and

X_1 and X_2 are independently, fluorine, chlorine, bromine, iodine, C1-C4 alkyl, NO_2 or

 .

3. The modified heptapeptide of Claim 2, wherein

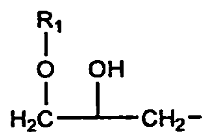
R_1 is



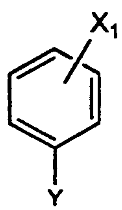
Y is CH_2 and

X_1 is hydrogen.

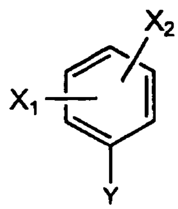
4. The modified heptapeptide of Claim 1, wherein A is



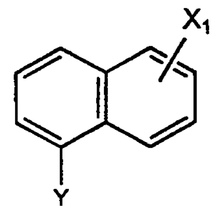
R1 is C1-C4 alkyl, adamantyl,



,



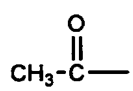
, or



,

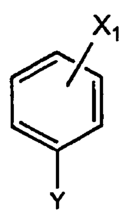
Y is a bond, C1-C4 alkenyl, C=O, SO_2; and

X_1 and X_2 are independently, fluorine, chlorine, bromine, iodine, C1-C4 alkyl, NO_2 or



.

5. The modified heptapeptide of Claim 4 wherein

R_1 is 

Y is a bond and X_1 is hydrogen.

6. A pharmaceutical composition comprising a compound of Formula I, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

7. A pharmaceutical composition comprising a modified heptapeptide of Claim 3, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

8. A pharmaceutical composition comprising a modified heptapeptide of Claim 5, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

9. A method for inhibiting the release of growth hormone, insulin, and glucagon in a mammal comprising administering to the mammal a modified heptapeptide of Formula I.

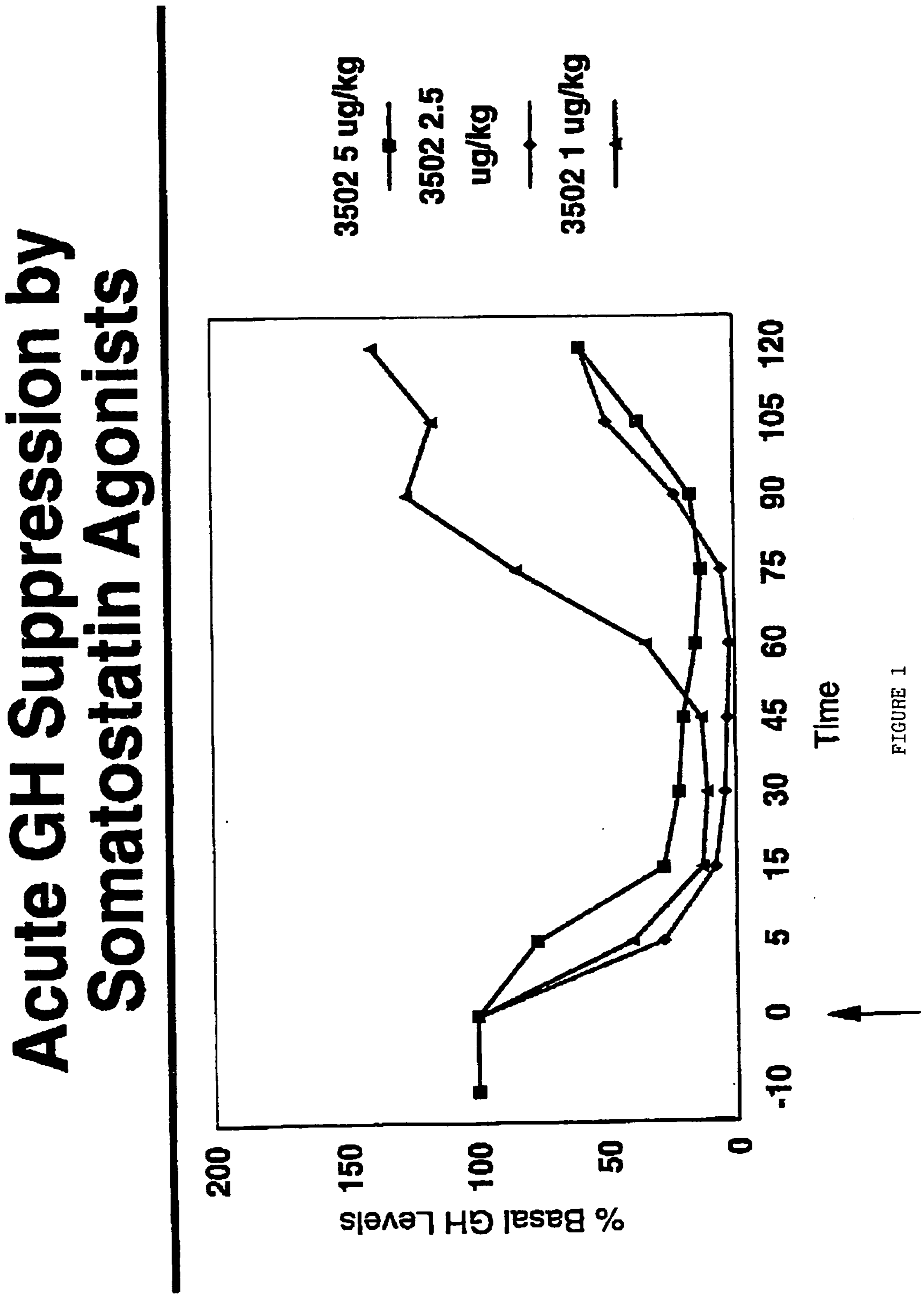

Acute GH Suppression by Somatostatin Agonists
% Basal GH Levels
200
150
100
50
0
-10
0
5
15
30
45
60
75
90
105
120
Time
3502 5 ug/kg
3502 2.5 ug/kg
3502 1 ug/kg

FIGURE 1

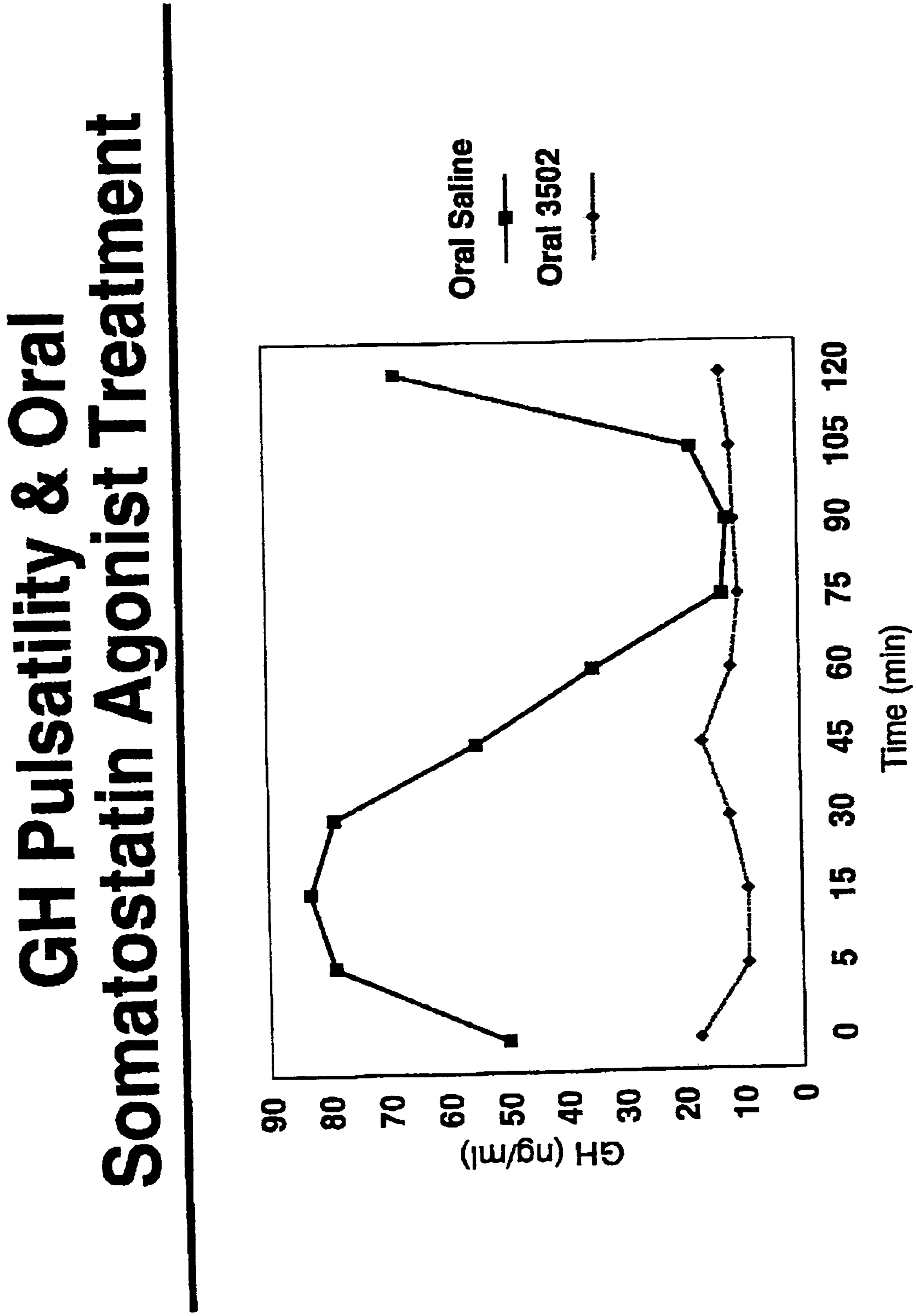

GH Pulsatility & Oral
Somatostatin Agonist Treatment
GH (ng/ml)
90
80
70
60
50
40
30
20
10
0
0
5
15
30
45
60
75
90
105
120
Time (min)
Oral Saline
Oral 3502

FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19090

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/00, 38/31, 38/12; C07K 5/00, 7/00
US CL :514/9, 11, 16; 530/300, 311, 317, 329
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/9, 11, 16; 530/300, 311, 317, 329

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, USPATFULL, BIOSIS, MEDLINE.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,145,337 A (DAIRMAN et al) 20 March 1979, see entire document.	1-9
A	US 5,770,687 A (HORNIK et al) 23 June 1998, see entire document.	1-9
A,P	US 5,846,934 A (BASS et al) 08 December 1998, see entire document.	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
20 DECEMBER 1999	11 JAN 2000

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